IMMUNOSTIMULATING COMPLEXES INCORPORATING *EIMERIA TENELLA* ANTIGENS AND PLANT SAPONINS AS EFFECTIVE DELIVERY SYSTEM FOR COCCIDIA VACCINE IMMUNIZATION

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ABSTRACT: Immunostimulating complexes (ISCOMs) are unique, multimolecular structures formed by encapsulating antigens, lipids, and triterpene saponins of plant origin, and are an effective delivery system for various kinds of antigens. The uses of ISCOMs formulated with saponins from plants collected in Kazakhstan, with antigens from the poultry coccidian parasite *Eimeria tenella*, were evaluated for their potential use in developing a vaccine for control of avian coccidiosis. Saponins isolated from the plants *Aesculus hippocastanum* and *Glycyrrhiza glabra* were partially purified by HPLC. The saponin fractions obtained from HPLC were evaluated for toxicity in chickens and chicken embryos. The HPLC saponin fractions with the least toxicity, compared to a commercial saponin Quil A, were used to assemble ISCOMs. When chicks were immunized with ISCOMs prepared with saponins from Kazakhstan plants and *E. tenella* antigens, and then challenged with *E. tenella* oocysts, significant protection was conveyed compared to immunization with antigen alone. The results of this study indicate that ISCOMs formulated with saponins isolated from plants indigenous to Kazakhstan are an effective antigen delivery system which may be successfully used, with low toxicity, for preparation of highly immunogenic coccidia vaccine.

Avian coccidiosis, an intestinal disease caused by intracellular coccidian protozoan parasites (*Eimeria* spp.), is estimated to cost the worldwide poultry industry over $1 billion annually through feed loss and lower bird productivity. The disease has been primarily controlled by use of anti-coccidial compounds that are mixed in the feed, but extensive use of these compounds over the past 40 yr has resulted in the inevitable development of drug resistance by these parasites (Yadav and Gupta, 2001; Allen and Fetterer, 2002; Williams, 2006). Many studies on the control of avian coccidiosis have centered on elicitation of protective immune response to parasite infection by development, delivery, and effective use of live, attenuated, or subunit recombinant parasite vaccines (Danforth et al., 1989, 1997; Jenkins, 1998; Dalloul and Lillehoj, 2005).

Immunostimulating complexes (ISCOMs) are one of the most successful delivery systems for various kinds of antigens of microbial, parasite, or viral origin. ISCOMs are a unique, multi-molecular structure formed by encapsulating antigens, lipids, and triterpene saponins of plant origin (Kensil, 1996; Morein and Abasugra, 2004). Previous studies have shown that ISCOMs incorporating Quil A, a saponin isolated from the bark of the South American tree *Quillaja saponaria*, or purified triterpene saponin QS-21 isolated from Quil A by HPLC fractionation, initiate a wide range of antigen-specific immune responses. Some of these include humoral and CD4/CD8 cell-mediated responses, stimulation of IL2 and IFN-gamma production, and mucosal immune response through subcutaneous, intranasal, and oral routes of immunization (Kensil, 1996; Agrawal et al., 2003; Morein and Abasugra, 2004). Various saponins, with complex-forming and immunostimulating activ-

ity similar to Quil A saponin, have been isolated from *Polygala senega*, a plant indigenous to Canada (Estrada et al., 2000).

It is proposed that ISCOMs made from plant saponins may be uniquely suitable for vaccine development because of their ability to elicit an effective, protective, low-toxicity immune response to coccidial antigens in birds. The purpose of the present research is the construction and validation of an effective ISCOMs delivery system for isolated *Eimeria* spp. antigens.

MATERIALS AND METHODS

Hosts and parasites

Chickens (Titan broilers) were purchased from local hatcheries. Feed and fresh water were given ad libitum. Before any experimental procedures were initiated, chickens were allowed to acclimate for 1 day. Chickens with a negative antibody titer for *E. tenella* antigens were transferred to separate housing for experimental procedures. Chickens used in all experiments were handled according to the guidelines of the Institutional Animal Care and Use Committee, Institute of Virology and Microbiology, Almaty, Kazakhstan.

Eimeria tenella (strain AK-1) were single oocyst-derived lines isolated from naturally infected chickens in Almaty, Kazakhstan, and were maintained at the Institute of Microbiology and Virology, Almaty, Kazakhstan by serial passage in 10- to 14-day-old chicks. Oocysts were produced and maintained by routine passage in 2-wk-old Titan broilers with an infection dose of 125,000 oocysts/chicken. Oocysts were collected from infected bird ceca, cleaned, sporulated, and excysted as previously described (Tomley, 1997; Fetterer and Barfield, 2003).

Eimeria tenella antigens

Crude *E. tenella* antigens were prepared by treating the coccidia suspension (a mixture of sporozoites, sporocysts, and oocysts) with 5% MESK, a novel non-ionic dialyzable detergent consisting of sugars and fatty acids (Berezin et al., 1988). After detergent treatment for 30 min at 4 C, the mixture was centrifuged at 10,000 *g* for 15 min at 4 C. Supernatant fluid was retained for preparation of ISCOMs as described below.

Saponin isolation

Saponins used in ISCOMs formation and immunological experiments were isolated from the plants *Aesculus hippocastanum* and *Glycyrrhiza glabra*, collected in mountainous areas of southeastern Kazakhstan. Crude saponins were obtained from roots and seeds by 95% ethanol extraction and then partially purified from low-weight substances by

extensive dialysis against phosphate-buffered saline. Plant extracts were lyophilized and fractionated by HPLC. Elution was performed with a linear gradient from a 0.1% trifluoroacetic acid (TFA) in water to an 80% acetonitrile (ACN) in water with 0.1% TFA. HPLC experiments were developed on a Waters (Milford, Massachusetts) chromatography system. Either an analytical column (Jupiter 5u C4 300A, 250 × 4.6 mm; Phenomenex, Torrance, California) or a semi-preparative column (Jupiter 5u C4 300A, 250 × 10.0 mm; Phenomenex) was used for separations. Absorbance was monitored at 208 nm and 353 nm using a photodiode array detector (Waters model 762). Aliquots (1 ml) were collected and pooled, as needed. Pooled fractions were dried under vacuum and stored at −20 C.

Toxicity studies

Toxicity of saponins was studied on 1-day-old chicks (broiler cross "Titan") and 9-day-old chick embryos. Crude saponin preparations and pooled HPLC fractions were tested in animals by administration of 0.2 ml intranasally in chickens or by inoculation into allantoic cavities of 9-day-old chick embryos. Toxicity of each saponin preparation was examined in a dose of 40–1,000 μg per animal. Toxicity of samples was determined by counts of dead animals or dead embryos 72 hr after inoculation of saponins, in each concentration. Hemolytic activity of saponin preparations was determined by measuring the release of hemoglobin from chicken erythrocytes (Kaler et al., 1986). Saponins were added in a 0.1-ml volume to 2 ml of 2% suspension of chicken erythrocytes, for a final concentration of 0.125 mg/ml, and incubated for 30 min at 37 C. After incubation with periodic shaking, the mixture was centrifuged at 1,000 g for 10 min. Released hemoglobin in supernatant was measured by spectroscopy at 412 nm. All solutions were prepared in isotonic buffer 140 mM NaCl, Tris-HCl buffer, pH 7.4; experiments were replicated 5 times.

ISCOM construction

ISCOMs were prepared using a dialysis technique. Isolated *E. tenella* antigens dissolved in 5% MESK detergent were mixed with saponins and lipids (lecithin from egg yolk) also dissolved in 5% MESK detergent. The mixture was subjected to extensive dialysis against phosphate-buffered saline. Removal of MESK detergent by dialysis results in formation of ISCOMs, which are self-assembling structures. ISCOMs particles, assembled with isolated coccidian antigens and various saponins, were studied by electron microscopy using a JEM-100B electron microscope (Jeol Ltd., Tokyo, Japan) with instrumental magnification of ×40,000–60,000. Samples of preparations were placed on formvare layer and negatively stained with 3% uranil acetate (Ozel et al., 1989). *Eimeria tenella* antigens, isolated from parasite membranes by MESK detergent extraction, were analyzed in 12.5% SDS-polyacrylamide gel (PAG) using a tris-glycine Laemmli system (Kensil et al., 1991). A ratio of 1:100 bis-acrilamide:acrilamide was used for gel preparation. Before gel electrophoresis, samples were treated with 2.5% SDS and 5% mercaptoethanol in a boiling water bath for 3 min (Laemmli, 1970). Protein concentration was determined by Bradford method using Coomassie blue stain. Optical density was measured at 595 nm (Bradford, 1976); 60–70 μg mixed proteins of *E. tenella* were loaded onto the gel. Commercial preparations of purified saponin Quil A (Super Fos Biosector, Fredricksound, Denmark) were also used for ISCOMs formation and for immunological experiments for saponins from Kazakstan plants, in the same manner described above. The preparations of plant saponins were free from proteins that may interfere with *E. tenella* antigens.

Challenge experiments

Two-wk-old chickens (7 birds per group) were immunized intranasally with various vaccine preparations containing *E. tenella* antigens. Two wk after immunization, all groups except the non-immunized, uninfected control group, were infected with live *E. tenella* at a dose of 50,000 oocysts per bird by oral gavage. Six days after infection, chickens were weighed and killed by cervical dislocation and the number of oocysts in the ceca counted.

Statistical analysis

The results are expressed as the mean ± SD. Statistical significance ($P < 0.05$) was determined using Student's 2-tailed t-test. Results are expressed as the mean ± SD of individual responses.
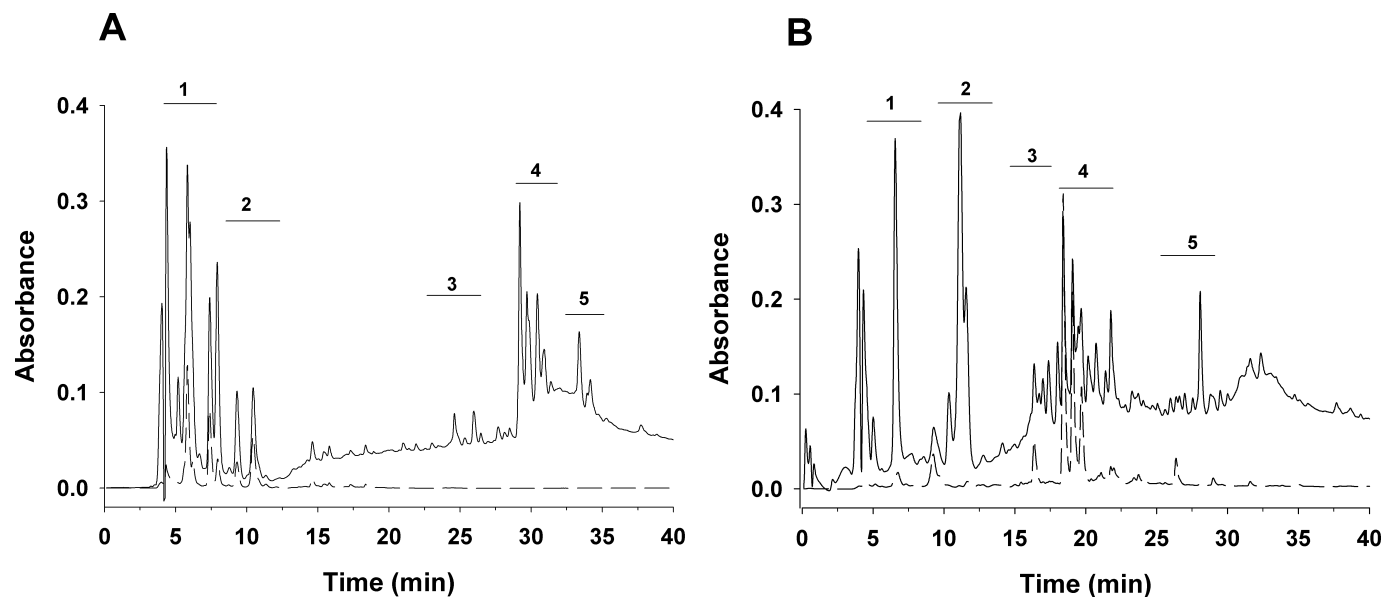

FIGURE 1.   HPLC fractionation of saponins extracted from *Aesculus hippocastanum* and *Glycyrrhiza glabra*. Saponins were separated by reverse-phase chromatography using a linear gradient. The optical density at 208 nm (solid line) and 353 nm (dashed line) was measured. *Aesculus hippocastanum* HPLC fraction nos. 5–8 were pooled as HPLC fraction no. 1; fraction nos. 9–12 were pooled as HPLC fraction no. 2; fraction nos. 24–27 were pooled as HPLC fraction no. 3; fraction nos. 29–32 were pooled as HPLC fraction no. 4; and fraction nos. 33–36 were pooled as HPLC fraction no. 5. *Glycrrhiza glabra* HPLC fraction nos. 6–8 were pooled as HPLC fraction no. 1; fraction nos. 10–13 were pooled as HPLC fraction no. 2; fraction nos. 15–18 were pooled as HPLC fraction no. 3; fraction nos. 20–23 were pooled as HPLC fraction no. 4; and fraction nos. 25–30 were pooled as HPLC fraction no. 5.
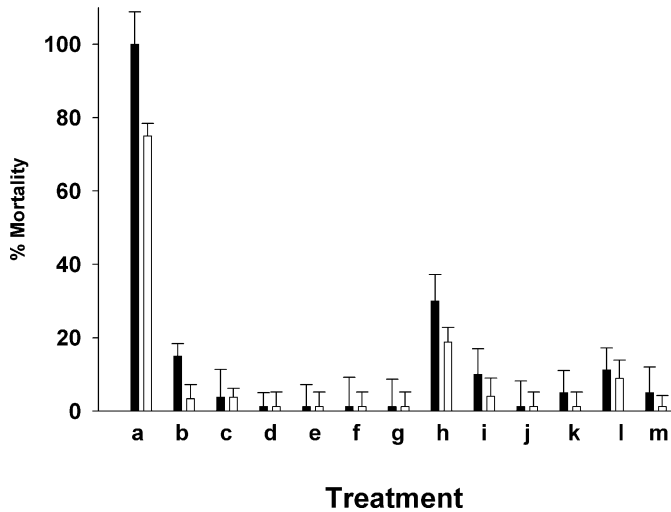
FIGURE 2. Comparative study of toxicity of various saponin preparations in doses of 1,000 μg per chick (closed bars) or 40 ug per chick embryo (open bars). (**a**) Commercial preparation of saponin Quil A; (**b**) non-fractionated *G. glabra* saponins; (**c**) *Glycrrhiza glabra* pooled HPLC fraction no. 1; (**d**) *Glycrrhiza glabra* pooled HPLC fraction no. 2; (**e**) *Glycrrhiza glabra* pooled HPLC fraction no. 3; (**f**) *Glycrrhiza glabra* pooled HPLC fraction no. 4; (**g**) *Glycrrhiza glabra* pooled HPLC fraction no. 5; (**h**) Non-fractionated saponins isolated from *A. hippocastanum*; (**i**) *Aesculus hippocastanum* pooled HPLC fraction no. 1; (**j**) *Aesculus hippocastanum* pooled HPLC fraction no. 2; (**k**) *Aesculus hippocastanum* pooled HPLC fraction no. 3; (**l**) *Aesculus hippocastanum* pooled HPLC fraction no. 4; (**m**) *Aesculus hippocastanum* pooled HPLC fraction no. 5. The results are expressed as the mean ± SD of individual responses. Values are means of 6–7 animals per group. Toxicity was determined as the number of dead/number of live animals or embryos 72 hr after inoculation and expressed as a percentage.

FIGURE 3. Hemolytic activity of saponins in a concentration of 2.5 mg/ml. (**a**) Commercial preparation of saponin Quil A; (**b**) non-fractionated *G. glabra* saponins; (**c**) *Glycrrhiza glabra* pooled HPLC fraction no. 1; (**d**) *Glycrrhiza glabra* pooled HPLC fraction no. 2; (**e**) *Glycrrhiza glabra* pooled HPLC fraction no. 3; (**f**) *Glycrrhiza glabra* pooled HPLC fraction no. 4; (**g**) *Glycrrhiza glabra* pooled HPLC fraction no. 5 (**h**) non-fractionated saponins isolated from *A. hippocastanum*; (**i**) *Aesculus hippocastanum* pooled HPLC fraction no. 1; (**j**) *Aesculus hippocastanum* pooled HPLC fraction no. 2; (**k**) *Aesculus hippocastanum* pooled HPLC fraction no. 3; (**l**) *Aesculus hippocastanum* pooled HPLC fraction no. 4; (**m**) *Aesculus hippocastanum* pooled HPLC fraction no. 5. The results are expressed as the mean ± SD of individual responses from 5 separate experiments. Values are means of absorbance at 412 nm.

RESULTS

Saponin isolation

The results of HPLC fractionation of saponin extracts prepared from *A. hippocastanum* and *G. glabra* plant tissues are presented in Figure 1. Numerous peaks at 208 nm, which is the maximum absorbance for saponins, were observed. A lesser number of peaks were observed at 353 nm (maximum absorbance for flavonoids and carotenoids). HPLC fractions containing saponins were pooled, as indicated in Figure 1.

Saponin toxicity

Plant extracts and pooled HPLC fractions were collected and their toxicity was determined. The results of studies in chickens and chick embryos demonstrated that saponins isolated from both plant species were generally of lower toxicity when compared with Quil A (Fig. 2). However, the saponins from both the extract and the HPLC fractions of *G. galabra* had the lowest toxicity when compared to Quil A. For both *A. hippocastanum* and *G. glabra*, the HPLC fractions had lower toxicity than crude plant extracts.

Saponin preparations isolated from the 2 plant species collected in Kazakhstan had lower hemolytic activity than commercial preparations of Quil A (Fig. 3). Similar to the toxicity assay, extracts and HPLC fractions from *G. glabra* had lower hemolytic activity compared to extracts or fractions from *A.*
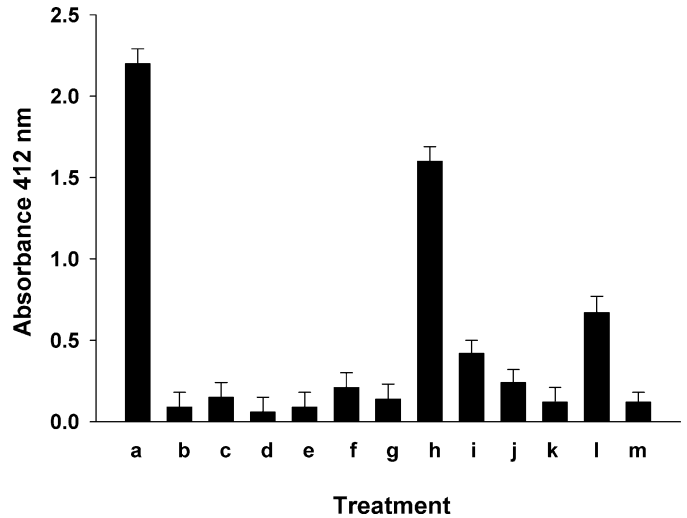
hippocastanum, and HPLC fractions from both plants had lower hemolytic activity than corresponding extract.

ISCOMs structure

The results of electrophoresis analysis showed that an extract of *E. tenella* antigens possessed a number of proteins with molecular weights from 160 kDa to 18kDa (Fig. 4). ISCOMs assembled with isolated *E. tenella* antigens and saponins purified by HPLC fractionation from *A. hippocastanum* (HPLC fraction no. 4) and *G. glabra* (HPLC fraction no. 2) were studied by electron microscopy. ISCOMs incorporating saponins isolated from native plants demonstrated the characteristic cage-like structure, about 40–60 nm in size (Figs. 5A, B). ISCOMs assembled with Quil A saponin exhibited similar particles, about 50 nm in size, when examined by electron microscopy (Fig. 5C).

Challenge experiments

Birds immunized with ISCOMs induced a greater level of protection against challenge with live *E. tenella* than with parasite antigens alone (Fig. 6). Immunization with ISCOMs containing Quil A or *G. glabra* saponins prevented daily weight decrease (Fig. 6A) and stimulated a decreased number of *E. tenella* oocysts in the ceca of immunized chickens (Fig. 6B).

DISCUSSION

There remains a need for treatments of low toxicity, treatments that do not rely on live parasites for a vaccine to protect
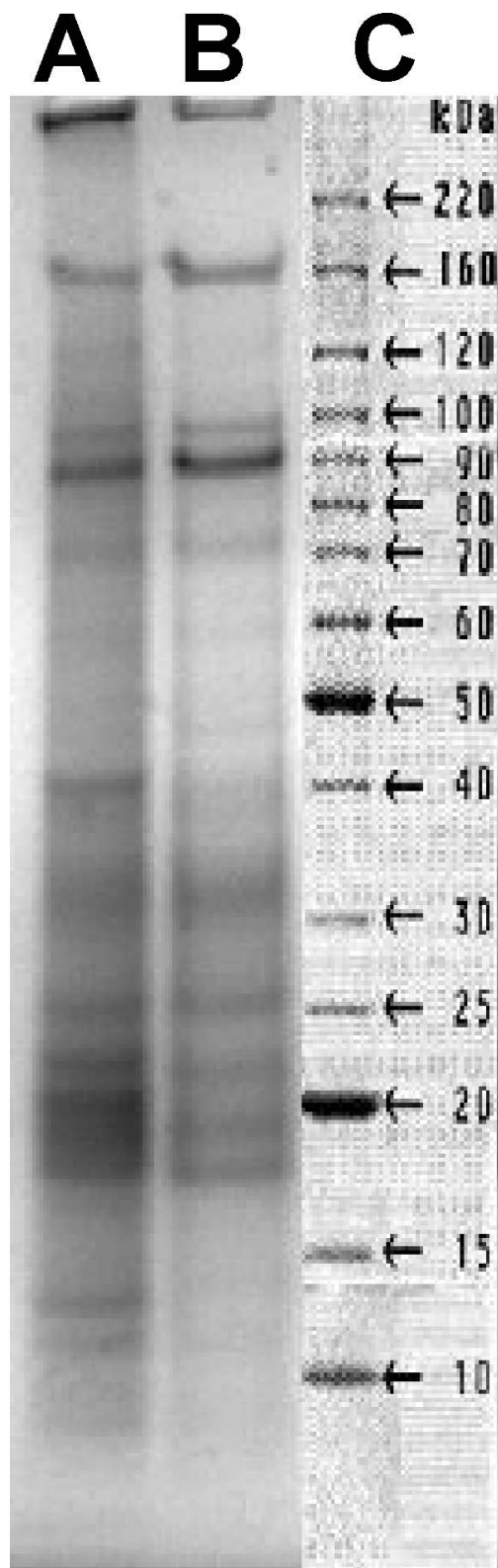
FIGURE 4. Electrophoretic analysis of *E. tenella* crude antigens isolated by MESK detergent extraction. (**A**) *Eimeria tenella* cell extract without MESK. (**B**) MESK isolated antigens. (**C**) Molecular weight markers. A mixture of purified *E. tenella* sporozoites, sporocysts, and oocysts was used for extraction of coccidian antigens.
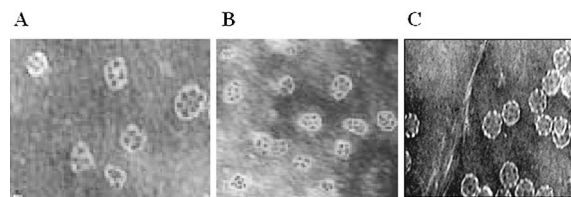

FIGURE 5. Electron microscopy study of complexes formed by saponins of different origin. (**A**) Saponins isolated from *G. glabra* (HPLC fraction no. 2). (**B**) Saponins isolated from *A. hippocastanum* (HPLC fraction no. 4). (**C**) Quil A.

chickens against economic losses due to avian coccidiosis. However, subunit vaccines, based on natural antigens isolated from the parasite or recombinant antigens, have been shown in birds to stimulate only partial protection against parasite chal-
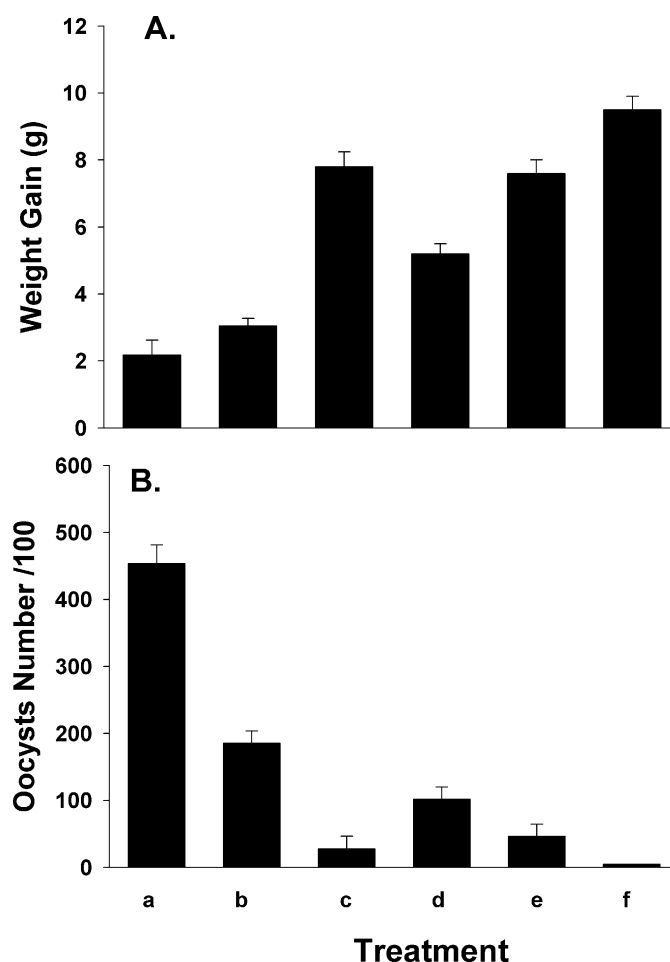

FIGURE 6. Protective activity of ISCOMs containing *E. tenella* antigens and *G. glabra* saponins. (**A**) Average daily chicken weight increase after *E. tenella* infection with a dose of 50,000 oocysts per chicken. (**B**) Cecal oocysts after immunization with ISCOMs; (**a**) non-immune infected chickens; (**b**) infected chickens immunized with isolated *Eimeria* antigens; (**c**) infected chickens immunized with ISCOMs incorporating Quil A; (**d**) infected chickens immunized with ISCOMs incorporating *G. glabra* saponins (HPLC fraction no. 2); (**e**) infected chickens immunized with ISCOMs incorporating *A. hipocastanum* saponins (HPLC fraction no. 4); (**f**) non-immune, uninfected chickens. The results are expressed as the mean ± SD of individual responses. Values are means for 7 chickens per group.

lenge (Murray et al., 1986; Danforth et. al., 1989; Crane et al., 1991; Jenkins, 1998). This emphasizes the need for improvement in the method to elicit a protective immune response.

To effectively deliver parasite antigens, ISCOMs should be assembled with saponins that will physically support the IS-COMs structure, while demonstrating low toxicity. The IS-COMs should also stimulate host immune responses and, most importantly, they must protect the host against challenge infection. Our current results indicate that ISCOMs made with saponins from native plants that incorporate *E. tenella* antigens meet the above criteria.

Our current results demonstrate that partially purified saponins from the 2 plant species collected in Kazakhstan can be used to form the ISCOM structure. In addition, the partially purified saponins collected in pooled HPLC fractions have a significantly lower toxicity, as measured by both reduction in mortality and hemolytic activity when compared to unfractionated Quil A, making them quite suitable for ISCOM construction. Of the 2 plants studied, the extract and fractions of *G. glabra* appear to have the lowest toxicity and therefore, may be the best saponin candidate for ISCOM construction.

Immunization of chickens with ISCOMs, prepared as described, offered protection to challenge infection, suggesting that ISCOMs can be the basis of an antigen delivery system for a coccidial vaccine. In addition, the protection observed with ISCOMs was superior to that observed with antigen alone, which is indicative of the immunostimulatory effect of IS-COMs. The birds were protected against 2 major effects of a coccidial infection, i.e., a decrease in weight gain and in oocyst output. There are a number of other response parameters of coccidial infection, including malabsorption, anemia, oxidative stress, and antibody responses, that were not measured in the current study. These parameters will be monitored in future, larger-scale trials of ISCOM preparations. However, preliminary results suggest that ISCOMs elevate antibody levels in chickens and induce cytokine production in mice (V. Berezin, unpubl.).

Realistically, a coccidia vaccine will consist of 1 or more recombinant proteins rather than crude *E. tenella* proteins, as used in the current study. Our continuing goal is to evaluate recombinant proteins in the ISCOM delivery system in order to develop a practical coccidia vaccine.

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